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ENZYME VARIATION IN ASHMUNELLA LEVETTEI  
(BLAND) (GASTROPODA:POLYGYRIDAEE).

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ENZYME VARIATION IN ASHMUNELLA LEVETTEI

(BLAND) (GASTROPODA:POLYGYRIDAE)

by

Harold Lee Fairbanks

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A Dissertation Submitted to the Faculty of the

DEPARTMENT OF GENERAL BIOLOGY

In Partial Fulfillment of the Requirements  
For the Degree of

DOCTOR OF PHILOSOPHY  
WITH A MAJOR IN ZOOLOGY

In the Graduate College

THE UNIVERSITY OF ARIZONA

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THE UNIVERSITY OF ARIZONA  
GRADUATE COLLEGE

I hereby recommend that this dissertation prepared under my direction  
by Harold Lee Fairbanks

entitled Enzyme variation in *Ashmunella levettei* (Bland)  
(Gastropoda:Polygyridae)

be accepted as fulfilling the dissertation requirement for the Degree  
of Doctor of Philosophy.

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Dissertation Director

6 July 1979  
Date

As members of the Final Examination Committee, we certify that we have  
read this dissertation and agree that it may be presented for final  
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SIGNED: Harold L. Fairbanks

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## TABLE OF CONTENTS

	Page
LIST OF TABLES . . . . .	v
LIST OF ILLUSTRATIONS . . . . .	vi
ABSTRACT . . . . .	vii
1. INTRODUCTION . . . . .	1
Electrophoresis in Gastropoda . . . . .	2
Background of <u>Ashmunella levettei</u> (Bland) . . . . .	3
Statement of the Problem . . . . .	4
2. MATERIALS AND METHODS . . . . .	8
Collection Localities . . . . .	8
Collection and Maintenance of Animals . . . . .	11
Experimental Procedures . . . . .	11
Sample Preparation . . . . .	12
Electrophoresis . . . . .	12
Staining Procedures . . . . .	15
Scoring the Gels . . . . .	15
Shell Examination . . . . .	16
Internal Anatomy . . . . .	16
Statistical Analyses . . . . .	16
Electrophoretic Analyses . . . . .	17
Morphological Analyses . . . . .	18
3. RESULTS . . . . .	19
Electrophoretic Results . . . . .	19
Morphological Results . . . . .	32
4. DISCUSSION . . . . .	42
Genic Variation . . . . .	42
F Statistics Analysis . . . . .	45
Phylogeny and Taxonomy . . . . .	55
Summary and Conclusions . . . . .	61
LITERATURE CITED . . . . .	64

LIST OF TABLES

Table		Page
1.	Description of Population Localities . . . . .	9
2.	Enzymes Assayed and Enzyme-buffer System Combinations Used in Electrophoresis . . . . .	14
3.	Results of Enzyme Stain Tests . . . . .	20
4.	Frequency of Genotypes at Polymorphic Loci . . . . .	23
5.	Chi Square Values for Tests of Population Goodness of Fit . . . . .	24
6.	Allelic Frequencies at Polymorphic Loci . . . . .	26
7.	Chi Square Contingency Test Results . . . . .	27
8.	Genetic Properties of the Populations Sampled . . . . .	28
9.	Expected Frequency of Genotypes . . . . .	30
10.	F Statistics for <u>Ashmunella levettei</u> . . . . .	31
11.	Rogers' Similarity Estimates between Populations . . . . .	33
12.	Shell Measurements . . . . .	35
13.	Results of Analysis of Variance Tests . . . . .	37
14.	Habitat Data . . . . .	39
15.	Plant Species Present at the Collection Localities . . . . .	41
16.	Chi Square Values for Species Goodness of Fit . . . . .	44
17.	F Statistics for <u>Rumina decollata</u> . . . . .	49
18.	Genetic Properties of the Subspecies of <u>Ashmunella levettei</u> . . . . .	57

LIST OF ILLUSTRATIONS

Figure		Page
1.	Collection Localities of <u>Ashmunella levettei</u> in the Huachuca Mountains . . . . .	5
2.	<u>Ashmunella levettei</u> and Its Subspecies . . . . .	6
3.	Diagrams of Electrophoretic Patterns . . . . .	22
4.	Dendogram of Rogers' Similarity Estimates between Populations . . . . .	34
5.	Results of Student-Newman-Keuls Tests . . . . .	38
6.	Dendogram of Rogers' Similarity Estimates between Subspecies . . . . .	59

## ABSTRACT

A study of genic variation in the land snail Ashmunella levettei (Bland) was undertaken. Emphasis was placed on variation as determined by horizontal starch-gel electrophoresis of enzymes obtained from minced foot-muscle tissue. The shells and internal anatomies were studied to determine morphological variation. Statistical analyses included Chi square, analysis of variance, Wright's F statistics and Rogers' genetic similarity index.

The goals of this study were to determine the genic variability in A. levettei and to investigate possible causes of the observed variability. In addition, the taxonomy of the populations included in this study was investigated.

Good results were obtained using stain techniques for six different enzymes; glutamate oxalacetate transaminase (GOT), isocitrate dehydrogenase (IDH), phosphoglucomutase (PGM), glucose-6-phosphate dehydrogenase (G-6-PD),  $\alpha$ -glycerophosphate dehydrogenase ( $\alpha$ -GPD), tetrazolium oxidase (TO). Nine loci were detected, three of which (GOT-1, IDH-1 and PGM-2) were polymorphic.

The electrophoretic results indicated a large deficiency of heterozygotes in all populations. In addition, the mean heterozygosity per individual was very low for land snails (3.13%). Mean genetic diversity was 2.455.

Wright's  $\bar{F}_{is}$  values were very high (.7055 - .7401), and genetic heterogeneity ( $F_{st}$ ) was also high (.3496 - .5259). The  $F_{it}$  values ranged from .4986 to .7813. The mean genetic similarity was .9003.

Variation of shell morphology was clinal in terms of diameter of shell, height of shell and number of whorls. Three variations were found in terms of the number of apertural teeth, those populations with no teeth, those with four teeth and those with the tooth number variable between zero and four. No variation of the internal anatomy was detected.

Studies of Wright's F statistics indicated that a high level of inbreeding was the major factor contributing to the observed heterozygote deficiency.

Studies of the shell morphology indicated that the taxonomy of the subspecies of A. levettei should be changed. The subspecies bifurca and angigyra were synonymized with the nominate subspecies Ashmunella levettei levettei (Bland), the electrophoretic data supported this conclusion. In addition, the subspecies varicifera was elevated to the rank of species; Ashmunella varicifera (Ancey). The status of the subspecies heterodonta remained unchanged.

## CHAPTER 1

### INTRODUCTION

Hunter and Markert (1957) are responsible for the development of the techniques used to identify enzymes from tissue extracts, by combining electrophoresis with histochemical staining techniques. Shortly thereafter, Markert and Møller (1959), using these techniques, reported the existence of isozymes, or multiple molecular forms of one enzyme that are coded at different loci.

As electrophoretic techniques improved, and the number of enzyme stains increased, the use of electrophoresis increased rapidly. These techniques were used in population biology studies by Lewontin and Hubby (1966), Johnson et al. (1966) and Harris (1966). These investigators were attempting to measure genetic variation in natural populations by searching for allozymes, enzymes from a single locus which differ in electrophoretic mobility and whose segregational behavior in populations follows Mendelian patterns (Avice 1975). Since that time, population geneticists, evolutionists and taxonomists have used electrophoresis to study various aspects of genetic diversity (for reviews see Lewontin 1974; Markert 1975; Nei 1975).

Comparative studies, based upon electrophoretically determined enzyme variation, of closely related species have revealed distinct differences between the species studied (e.g., Hubby and Throckmorton 1968;

Johnson et al. 1972; Manwell and Baker 1963). These findings led to the increased use of electrophoresis in taxonomy.

In the taxonomic use of electrophoresis, raw data in the form of allelic frequencies are used to calculate a number which indicates the degree of genetic similarity (or distance) between two populations (see Nei 1972; Rogers 1972). The resulting phylogenetic schemes (dendograms) produced using these indices agree well with classical morphological methods (Avisé 1975).

#### Electrophoresis in Gastropoda

In general, the early electrophoretic studies involving gastropods were not population genetics nor taxonomic studies. The investigators were concentrating upon the identification of enzyme systems and studying the effects of parasites upon enzyme variability (Targett 1963; Wright and Ross 1965; Wright, File and Ross 1966; Davis 1969; Coles 1969, 1970; Burch and Lindsay 1968; Stadnichenko 1970; Oxford 1973).

The first studies that measured genetic diversity in natural populations of gastropods appeared about 1970 (e.g., Schwabl and Murray 1970). Since that time, several additional studies investigating genetic diversity and population structure of species of gastropods have appeared (e.g., Gooch, Smith and Knupp 1972; Wahren and Tegelstrom 1973; Selander and Kaufman 1973; Selander and Hudson 1976; Jarvinan et al. 1976; Selander, Parker and Browne 1978).

In addition, several taxonomic studies, utilizing electrophoresis, of different species of gastropods have been published (e.g., Gould, Woodruff and Martin 1974; Chambers 1978; Murphy 1978). None of

these studies nor any of the previously noted studies have involved species of land snails that are indigenous to the United States. Selander et al. (1978) studied variation in Campeloma decisa (Say 1819), a parthenogenetic aquatic snail found in the eastern United States. Geographic variation in North American populations of Cepaea nemoralis Linnaeus 1758 (Brussard 1975) and Helix aspersa Müller 1774 (Selander and Kaufman 1975) has been studied. Selander and Kaufman (1973) also investigated population structure in Rumina decollata (Linnaeus 1758). These latter studies used non-native land snails living in and around cities in which there was favorable habitat.

Background of Ashmunella levettei  
(Bland)

The genus Ashmunella (Pilsbry and Cockerell 1899) is included in the family Polygyridae. The shell is heliciform, umbilicate, unbanded and with or without apertural teeth. The genus is restricted to a relatively small area, with a present center of density located in south central New Mexico (Pilsbry 1940). Species of Ashmunella are also found in the northwestern corner of Texas, northern Chichuahua, and the southeastern corner of Arizona. Most of the 32 recognized species live at elevations of 6000-8000 feet, with some found as low as 4000 feet, others as high as 12,000 feet. They prefer well-sheltered and shaded habitat in sloping talus of loose soil and rock debris (Bequaert and Miller 1973).

The range of Ashmunella reaches its western-most point in the Huachuca Mountains, which are located in the southwestern corner of

Cochise County, Arizona. These mountains lie in a southeast to northwest line (Fig. 1), are well forested, and have several canyons in which water can be found year around. The highest point in the range is Miller Peak, 9466 feet, which is located near the southern end of the range. Pilsbry (1940) noted two species and five subspecies of Ashmunella from the Huachucas. At the present time, Bequaert and Miller (1973) recognize one species, A. levettei (Bland 1880) and four subspecies, A. l. angigyra Pilsbry 1905 , A. l. bifurca Pilsbry and Ferriss 1909 , A. l. heterodonta Pilsbry 1905 and A. l. varicifera (Ancey 1901).

Ashmunella levettei and its subspecies (Fig. 2) are generally found on the north facing slopes of a canyon, although at high elevations almost any talus slide will yield specimens. Generally, the habitat is sheltered, shaded and with a considerable amount of ground litter. Most of the populations are comprised of "toothed" shell individuals, i.e., have 1-4 lamellae in the aperture (Fig. 2). However there are populations of individuals with "untoothed" shells. The internal anatomy of these taxa is remarkably similar, which led Pilsbry (1940) to state that ". . . the genitalia, which are so much alike that it would be impossible to tell the species apart, . . .".

#### Statement of the Problem

Mayr (1970) wrote that evolutionary change is brought about by changing the frequency of genes in populations. Since speciation appears to be extensive among the various genera of large land snails found in the southwest United States, one might expect considerable genic variability within these groups. However, in many instances, the

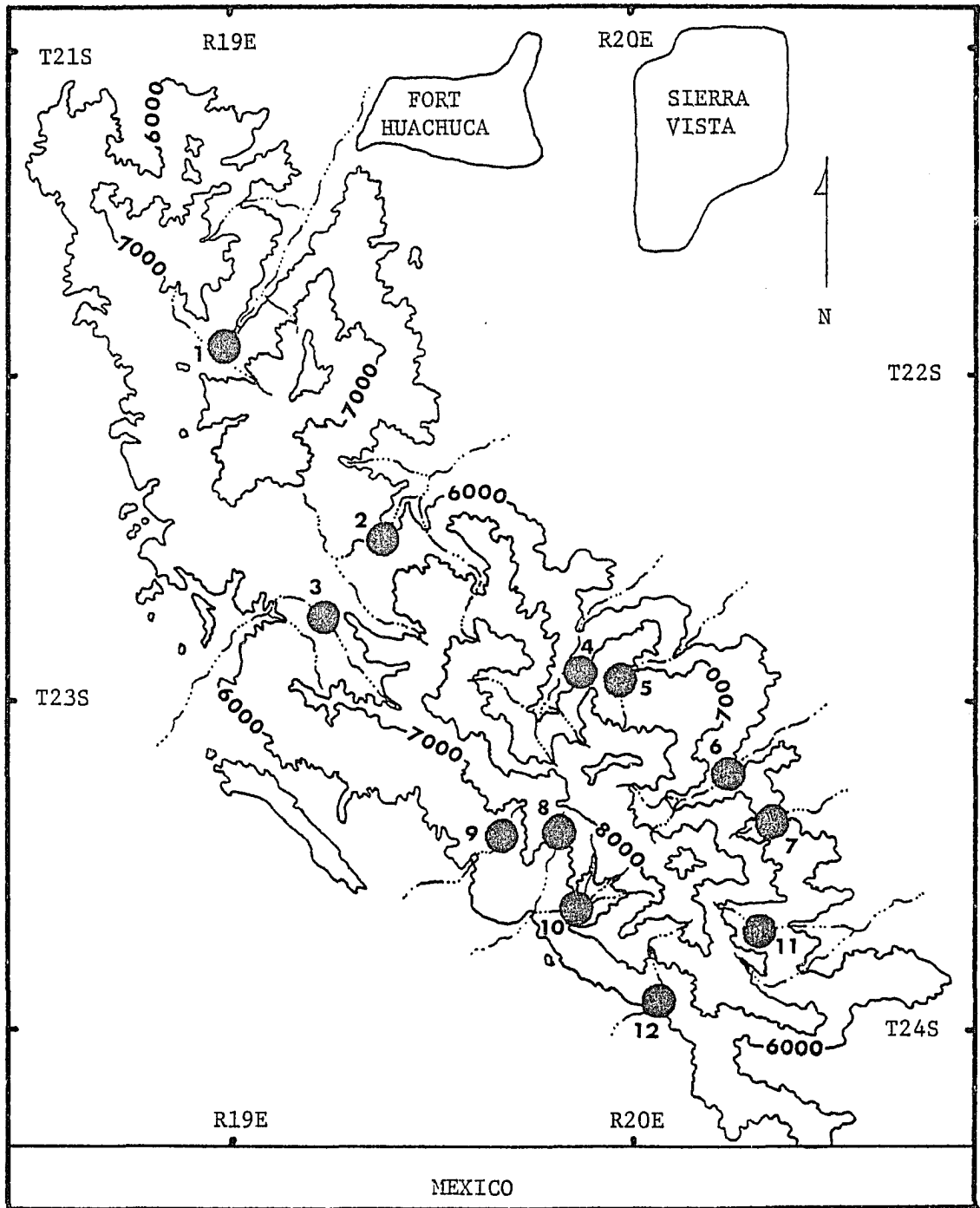


Figure 1. Collection Localities of *Ashmunella levettei* in the Huachuca Mountains.

Range and township are indicated along the borders.

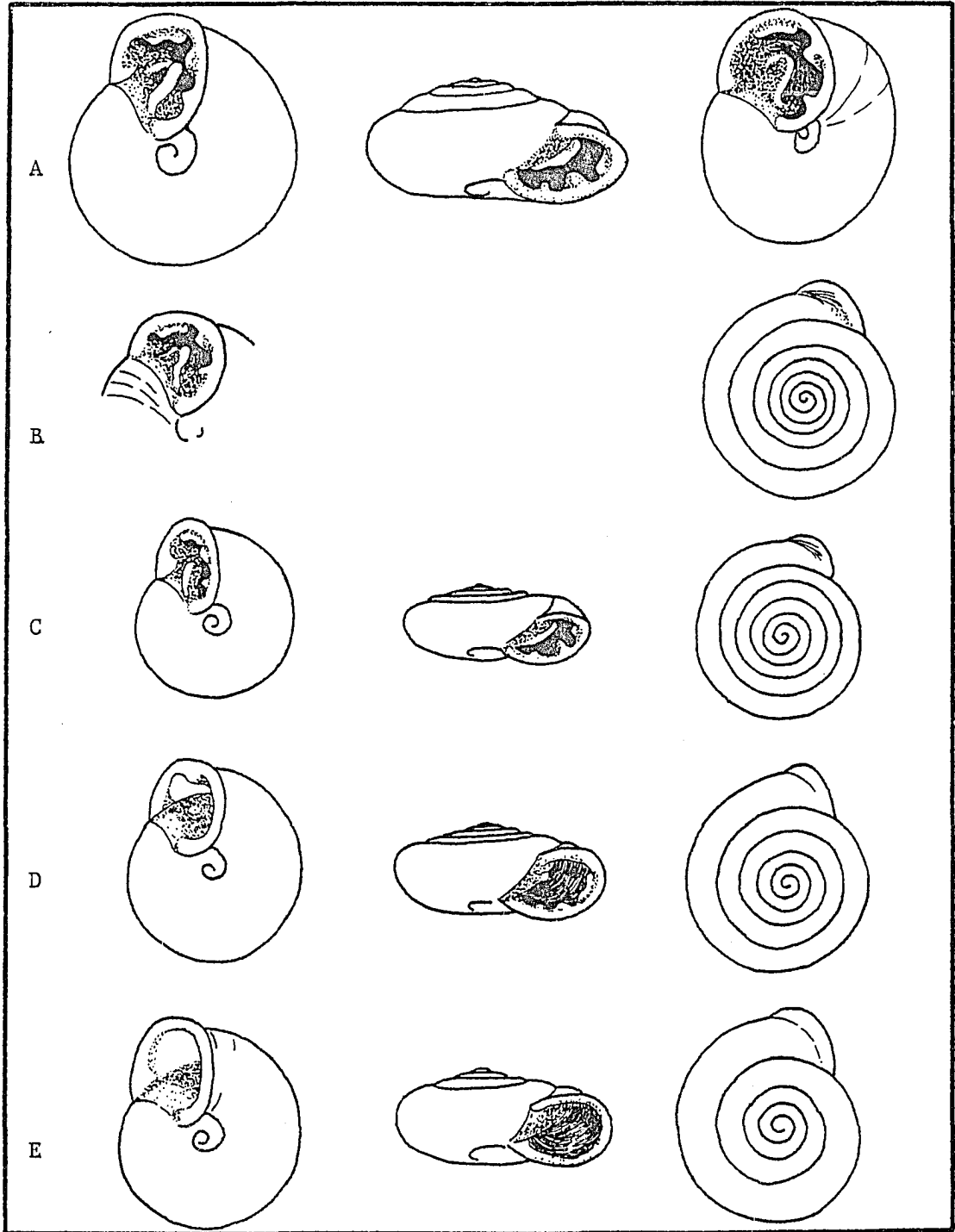


Figure 2. Ashmunella levettei and Its Subspecies.

- (A) A. l. levettei; (B) A. l. bifurca; (C) A. l. angigyra;  
 (D) A. l. heterodonta; (E) A. l. varicifera.

land snails of the southwest U.S. exist in small populations and thus might be expected to exhibit reduced genic variability (Selander 1976). No rigorous analysis has been conducted to determine genic variability within any of the land snail groups found in the southwest U.S., and thus the degree of genic variability among and within these groups is unknown.

Ashmunella levettei provides an opportunity to study the genic variability of a species in which morphological polymorphism is known, genic variability is unknown and the status of some of the taxa is in doubt.

Thus, the purpose of this study is to accomplish the following: (1) to determine, via electrophoresis, the genic variability within and among several populations of Ashmunella levettei; (2) to investigate possible causes of the observed variation; (3) to study the phylogenetic relationships between the populations studied; (4) to verify, or modify, current opinions on the speciation and taxonomy of these populations.

## CHAPTER 2

### MATERIALS AND METHODS

The organization of this section follows, essentially, the chronological sequence in which the various procedures were conducted. The subheadings reflect the various aspects of the study.

#### Collection Localities

All of the specimens utilized in this investigation were collected at twelve different localities in the Huachuca Mountains, Cochise County, Arizona (Fig. 1). Locality selection was based on the desire to cover the entire potentially habitable area (for Ashmunella) of the Huachuca Mountains, and in nearly all cases, was based on the prior knowledge of the presence of Ashmunella levettei. Table 1 lists the location of each of the twelve collection localities. The locations were verified by compass triangulation and altimeter reading, to reduce location error. In addition to location data, the following habitat data were also collected: exposure (determined by compass); microhabitat, i.e., type of cover; amount of ground litter; amount of shade; amount of moisture; size of area over which specimens were collected (the last four parameters were determined subjectively); vegetation present.

Table 1. Description of Population Localities.

All localities are located in the Huachuca Mountains, Cochise County, Arizona.

Population	Locale
1	Huachuca Canyon, 1.7 miles, by road, beyond the spring near the last picnic spot; on south side of creek; SW 1/4 of SW 1/4 of sec 22 T22S R19E at 6100 feet. C.C. Christensen, J.S. Frost, H.L. Fairbanks, 3 Aug 1978.
2	Garden Canyon, 1.65 miles, by road, beyond the upper picnic area; on south side of creek; NE 1/4 of SW 1/4 of sec 1 T23S R19E at 6100 feet. C.C. Christensen, J.S. Frost, H.L. Fairbanks, 3 Aug 1978.
3	Sunnyside Canyon, Collected along 1/2 mile of canyon, on west side; NW 1/4 of SE 1/4 of sec 11 and NW 1/4 of NW 1/4 of sec 13 T23S R19E at altitudes varying between 6100 feet and 6325 feet. J.S. Frost, H.L. Fairbanks, 1 Aug 1978.
4	Ramsey Canyon, above trail that goes from Comfort Spring to head of Ramsey Canyon; NW 1/4 of SE 1/4 of sec 16 T23S R20E at 7200 feet. J.S. Frost, H.L. Fairbanks, 26 Jul 1978.
5	Carr Canyon, west side of creek at junction with small stream that tumbles over a 15-20 foot high rock wall (rock wall about 100 feet from Carr Creek); NW 1/4 of SW 1/4 of sec 15 T23S R20E at 7025 feet. C.C. Christensen, H. L. Fairbanks, 16 Jul 1978.
6	Miller Canyon, at south side of collection site for Tombstone Waterworks; SE 1/4 of SW 1/4 of sec 23 T23S R20E at 6050 feet. J.S. Frost, H.L. Fairbanks, 18 Jul 1978.
7	Hunter Canyon, south side of stream, at end of road (concrete basin on north side of stream); NW 1/4 of SW 1/4 of sec 25 T23S R20E at 6200 feet. E.S. Fairbanks, H.L. Fairbanks, 5 Aug 1978.

Table 1. (Continued)

Population	Locale
8	Ida Canyon, 1.3 miles, by road, from junction with Cave Canyon; NW 1/4 of SW 1/4 of sec 28 T23S R20E at 7025 feet. C.C. Christensen, H.L. Fairbanks, 30 Jul 1978.
9	Bear Canyon, 2.6 miles, by road, from main road along west side of Huachuca Mts.; SE 1/4 of SW 1/4 of sec 29 T23S R20E at 6200 feet. C.C. Christensen, J.S. Frost, H.L. Fairbanks, 8 Aug 1978.
10	Cave Canyon, south side of canyon 1/4 mile beyond mines; SE 1/4 of SW 1/4 of sec 33 T23S R20E at 6425 feet. C.C. Christensen, H.L. Fairbanks, 24 Jul 1978.
11	Lutz Canyon, south side of road about 1/4 mile beyond junction of Lutz Creek and Ash Creek; collected along 1/4 mile of canyon; NW 1/4 of NW 1/4 of sec 1 and NE 1/4 of NE 1/4 of sec 2 T24S R20E at altitudes varying between 6200 feet and 6400 feet. C.C. Christensen, H.L. Fairbanks, 20 Jul 1978 and E.S. Fairbanks, H.L. Fairbanks, 5 Aug 1978.
12	Copper Canyon, at Eighty Spring, just south of the mine, about 100 feet from the road; NE 1/4 of NW 1/4 of sec 10 T24S R20E at 6100 feet. J.S. Frost, H.L. Fairbanks, 28 Jul 1978.

### Collection and Maintenance of Animals

At each collection locality, search for specimens was concentrated upon the areas beneath rocks and/or logs. In some especially moist localities, specimens were collected while they were crawling along the ground. The number of specimens collected was arbitrarily set at 25-30 for two reasons. First, many populations were small and thus the possibility of seriously depleting them existed. It was decided that a sample of 25-30 would not harm the small populations. Second, Davis and Lindsay (1967) determined that 10 specimens would detect all but the very rare alleles. It was decided that 20 specimens would detect even rare alleles and provide a statistically significant sample size.

As they were collected, the snails were placed in a one pint plastic container that was one half filled with litter from that collection locality. On arrival at The University of Arizona, the specimens, along with the litter, were placed in a terrarium. The terraria were housed in a room in which the temperature was maintained 14°C to 18°C and the relative humidity was kept above 40%. The snails were allowed to estivate until they were prepared for use in electrophoresis.

### Experimental Procedures

The following section presents the electrophoretic procedures and apparatus used in this study. Not included is an outline of the preliminary work conducted to determine the best buffer-stain combinations to use with Ashmunella levettei.

### Sample Preparation

The procedures used for sample preparation are essentially the same as those used by Brussard and McCracken (1974), with the following modifications. The enzymes were "leached" from the minced foot-muscle tissue in glass distilled water rather than a buffer solution. In addition, sonication of the tissue sample was not used to "free" the enzymes from the cells. Instead, good results were obtained by allowing the sample to sit at room temperature (22°C) for one hour (Selander and Hudson 1976). Centrifugation was conducted at 17,000 RPM at 4°C for 20 minutes.

### Electrophoresis

The gel chamber, buffer trays and power supply connections were as described by Utter, Hodgins and Allendorf (1974). Deviations from their procedures were minor. First, all tests were conducted inside a refrigerator at 4°C. Before they were used, the gels were allowed to cool at room temperature (22°C) for one half hour and then cooled for an additional one half hour inside the refrigerator at 4°C. Sample inserts were left in the gel for the entire test, rather than removing them after ten minutes.

Three buffer systems and eight enzyme stains were used in the course of this study. Buffer systems used were:

A. Tris-citrate      pH 7.0

0.135 M Tris  
0.043 M Citrate

source: Siciliano and Shaw 1978

## B. Tris-versene-borate      pH 8.0

0.5M Tris  
0.016M Versene  
0.65M Borate

source: Siciliano and Shaw 1978

## C. Lithium hydroxide

a. pH 8.1  
0.03M Lithium hydroxide  
0.19M Boric acid

b. pH 8.4  
0.05M Tris  
0.008M Citric acid

source: Selander et al. 1971

See Table 2 for enzymes and enzyme-buffer system combinations used in this investigation.

Gels were prepared using 36g of Electro-starch (Electro-starch Co., P.O. Box 1294, Madison, Wisconsin 53701) in 300 ml of the appropriate buffer. The Electro-starch was placed in a 1000 ml Erlenmeyer flask, and 300 ml of buffer solution was added. The mixture was swirled vigorously until the Electro-starch was suspended in the buffer. With continued swirling, the mixture was then heated until cooked to a viscous opaque mass. The mixture was then degassed for one minute, to remove air bubbles, and then poured into a gel chamber. A plexiglass sheet was carefully placed upon the gel chamber to squeeze out excess gel material. Weights were set on the plexiglass sheet to hold it in place. After 30 minutes, the plexiglass sheet was removed, excess material was trimmed from the edges of the gel and then Saran Wrap was used to cover

Table 2. Enzymes Assayed and Enzyme-buffer System Combinations Used in Electrophoresis.

Enzyme assayed	Abbreviation	Buffer system	Source
Glucose-6-phosphate dehydrogenase	G-6-PD	B	Siciliano & Shaw 1978
Glutamate oxalacetate transaminase	GOT	C	Siciliano & Shaw 1978
$\alpha$ -Glycerophosphate dehydrogenase	$\alpha$ -GPD	B	Siciliano & Shaw 1978
Isocitrate dehydrogenase	IDH	B	Siciliano & Shaw 1978
Leucine aminopeptidase	LAP	B	Selander et al. 1971
Malate dehydrogenase	MDH	A	Selander et al. 1971
Phosphoglucomutase	PGM	A	Siciliano & Shaw 1978
Tetrazolium oxidase	TO	B	Siciliano & Shaw 1978

the gel. The gel-gel chamber combination was then placed in the refrigerator at 4°C to cool for an additional 30 minutes.

A filter paper insert (Whatman No. 1) three millimeters by six millimeters, dipped in the supernatant of a tissue sample and blotted to remove excess fluid, was placed in a cut in the gel 4.0 cm from the cathodal end of the gel. Twenty-five specimens were tested on each gel. Voltage was applied until the marker dye had moved 6-8 cm from the origin.

#### Staining Procedures

The gels were prepared for staining as described in Utter et al. (1974). The only modification was the use of stainless steel wire, instead of nylon thread, to slice the gels. Staining procedure was as described in Siciliano and Shaw (1978). Incubation of stains was conducted in the dark at 37°C. Stain time varied from twenty minutes to one hour depending upon which stain was being used.

#### Scoring the Gels

As soon as staining was completed, the stained gel was rinsed three times in tap water and then photographed. The system used to score the gels was as described by Woodruff (1975). The alleles segregating at a given locus were treated as codominant. After completion of the scoring, a written record was made of each gel. The gel slice was then fixed (Siciliano and Shaw 1978), wrapped in Saran Wrap and stored in a refrigerator.

The decision concerning which stains to utilize was based upon the following criteria. First, the electromorphs (alleles) at each locus had to be clearly distinct from one another so that reliable and consistent scoring could be conducted. Second, each enzyme system used had to be scorable in all populations.

#### Shell Examination

The shells of all specimens collected at each locality, both alive and dead, were examined. Shells that were broken during freezing or sample removal, were not included in this part of the study. The following data were obtained for each specimen: diameter of shell; height of shell; umbilicus diameter; number of whorls; number of teeth in the aperture. Measurements were taken using a vernier caliper or ocular micrometer.

#### Internal Anatomy

Three representatives of each population sampled were dissected. Visual comparisons were made of the kidney, ureter, mantle surface (lung), nerve ganglia (brain), heart and ovotestis. Then the entire reproductive system, except for the ovotestis, of each specimen was removed, stained and cleared, and then mounted on a glass slide for examination.

#### Statistical Analyses

Two sets of statistical analyses were made, namely, those analyses dealing with the results of the electrophoretic tests, and those analyses treating the morphological data.

## Electrophoretic Analyses

Genotypic and allelic frequencies for each population and for the species as a whole were determined by direct count. Expected genotypic frequencies based on Hardy-Weinberg were calculated using Levene's (1949) formula for small samples. Chi square analysis was used to test within population genotypic frequencies for goodness of fit to Hardy-Weinberg expectations. The chi square is a test of the null hypothesis that the observed genotypic frequencies do not differ significantly from those predicted by Hardy-Weinberg equilibrium.

A chi square contingency test was used to test for heterogeneity in variation of genotypic and allelic frequencies among populations. This analysis tests the null hypothesis that frequencies between populations do not deviate more than would be expected by chance alone.

Estimates of the average proportion of the genome heterozygous per individual were obtained by direct count and by calculation based on allelic frequencies (Selander 1976).

Genetic diversity was calculated using the Shannon information measure as described by Lewontin (1972).

Analysis of population structure was conducted using Wright's F statistics (S. Wright 1943, 1951, 1965, 1978). This group of statistics is informative since the correlation between uniting gametes is partitioned into within group, between groups and total correlations, thus providing information about all three levels of population structure (Schaal 1975).

Rogers' (1972) genetic similarity index was calculated by Dec-10 computer, the program was provided by F. W. Allendorf, University of Montana, Missoula, Montana 59801. A dendogram was constructed (un-weighted average linkage method) as described by Bailey (1967, p. 150).

#### Morphological Analyses

Shell morphology was analyzed by use of analysis of variance (Sokal and Rohlf 1969, p. 218) and the Student-Newman-Keuls test (Sokal and Rohlf 1969, p. 240). Internal anatomies were compared visually.

## CHAPTER 3

### RESULTS

In this study variation at two levels, cryptic (electrophoretic) and noncryptic (morphological) was investigated. Hence, it seems appropriate to present the resulting data in two sections. Most of the data is presented in tabular form. The habitat data are presented at the end of the morphological analyses.

#### Electrophoretic Results

Initial testing involved eight enzyme systems (Table 2). These tests detected 14 loci, with five of these loci polymorphic (more than one allele) (Table 3). Based on 14 loci, the Ashmunella levettei populations sampled had 35.7% of their loci polymorphic. Unfortunately, there were problems concerning two of the polymorphic loci (LAP-2 and MDH-3).

Polymorphism was observed at the LAP-2 locus, but no heterozygotes were detected despite the presence of two different alleles (i.e., homozygotes for each allele) in each of seven populations. Alex Kahler (Research Associate, University of California at Davis; personal communication, 27 March 1979) suggested the possibility of a dominant gene masking the heterozygous individual, although evidence of this among enzyme loci, is very rare. Accordingly, the lack of heterozygotes dictated that the LAP systems be dropped from the study.

Table 3. Results of Enzyme Stain Tests.

Enzyme	Number of loci detected	Number of loci polymorphic	Number of alleles at polymorphic locus
GOT	2	1	2
IDH	2	1	2
PGM	2	1	3
LAP	2	1	2
MDH	3	1	2 ?
G-6-PD	1	0	-
$\alpha$ -GPD	1	0	-
TO	1	0	-

At the MDH-3 locus, the allelic patterns could not be scored. At each of the three loci detected, multiple banding was observed. The number of bands observed was not consistent, preventing reliable scoring. The multiple banding was probably the result of small molecule binding (Levan and Fredga 1972), i.e., the enzymes bind one or more molecules from the buffer or gel. A difference in the number of bound molecules results in a multiple band pattern. Accordingly, the MDH loci were dropped from further study.

Fig. 3 gives examples of the zymogram patterns observed for the polymorphic loci studied. Both GOT-1 and IDH-1 had two alleles segregating, whereas PGM-2 had three alleles segregating. The alleles segregating at a locus were assumed to be codominant. Although no tests involving Ashmunella levettei have been conducted to substantiate this assumption Brussard and McCracken (1974) showed that the alleles segregating at two loci in Cepaea nemoralis were codominant.

The observed genotypic frequencies of the polymorphic loci are listed in Table 4. The data show that four populations (1, 2, 3, 9) were fixed for the "b" allele (i.e., all bb homozygotes) at GOT-1. Population 12 was fixed for the "a" allele at GOT-1. Populations 4 through 8 and 10 and 11 were polymorphic at GOT-1. Chi square tests for goodness of fit to Hardy-Weinberg expectations indicated that the genotypic frequencies in populations 4, 5 and 7 were significantly different from H-W expectations (Table 5).

At the IDH-1 locus, only three populations were polymorphic (Table 4). All other populations were fixed for the "b" allele. Only

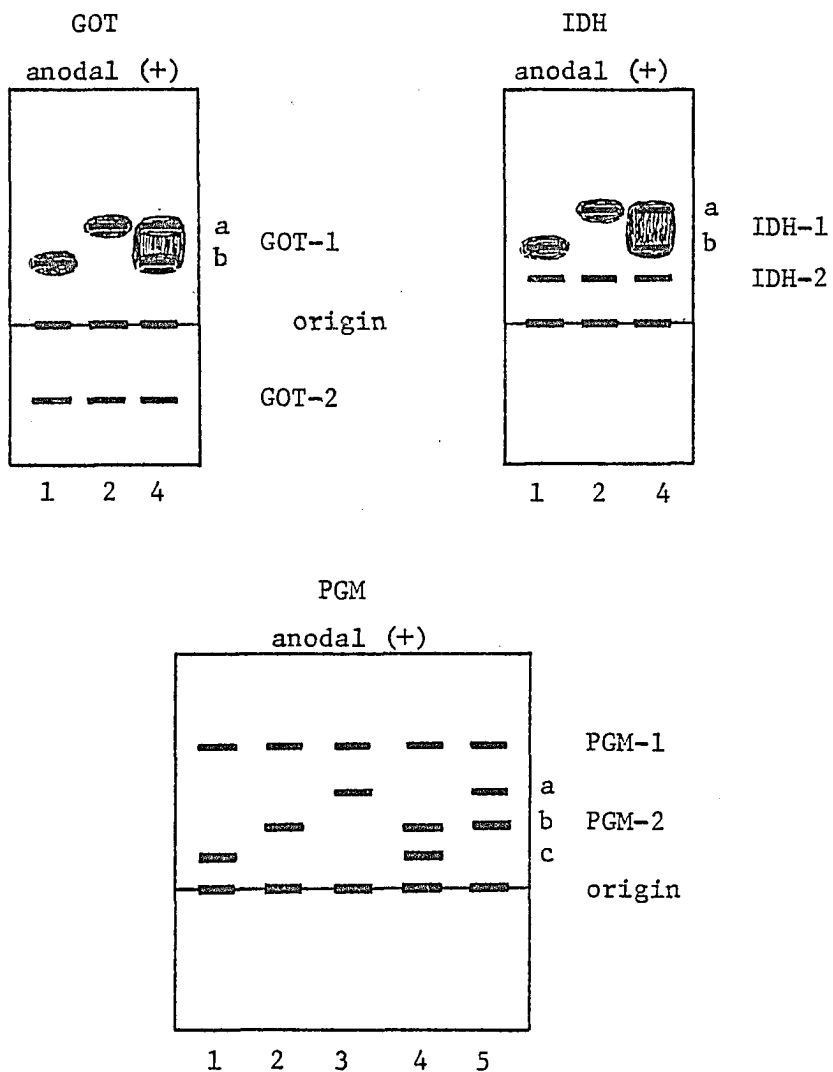


Figure 3. Diagrams of Electrophoretic Patterns.

Shown are the three systems having polymorphic loci, all other enzyme systems were monomorphic. In each pattern, 1, 2, and 3 (if present) are homozygotes; 4 and 5 are heterozygotes. No PGM-2 a-c heterozygotes were observed.

Table 4. Frequency of Genotypes at Polymorphic Loci.

Population	N	GOT-1			N	IDH-1			N	PGM-2					
		aa	ab	bb		aa	ab	bb		aa	ab	bb	ac	bc	cc
1	20	-	-	20	18	-	-	18	20	-	-	3	-	-	17
2	19	-	-	19	18	-	-	18	20	-	-	6	-	4	10
3	23	-	-	23	13	-	-	13	23	-	-	19	-	2	2
4	20	4	3	13	12	-	-	12	20	-	-	15	-	1	4
5	20	6	5	9	14	-	-	14	20	4	4	8	-	-	4
6	20	1	4	15	13	-	-	13	20	-	-	20	-	-	-
7	20	4	2	14	10	-	-	10	20	-	-	20	-	-	-
8	19	3	2	14	18	7	9	2	19	3	3	13	-	-	-
9	19	-	-	19	20	3	4	13	20	2	2	16	-	-	-
10	20	9	8	3	20	1	5	14	20	-	-	16	-	4	-
11	20	18	-	2	19	-	-	19	20	-	-	7	-	3	10
12	20	20	-	-	20	-	-	20	19	-	-	17	-	-	2
Totals	240	65	24	151	195	11	18	166	241	9	9	160	0	14	49

Table 5. Chi Square Values for Tests of Population Goodness of Fit.

Population	X <sup>2</sup>	GOT-1		X <sup>2</sup>	IDH-1		X <sup>2</sup>	PGM-2	
		df	Probability		df	Probability		df	Probability
1	-	-	-	-	-	-	1.676	1	>.100
2	-	-	-	-	-	-	7.331	1	<.010*
3	-	-	-	-	-	-	0.674	1	>.100
4	8.780	1	<.005*	-	-	-	1.914	1	>.100
5	5.250	1	<.025*	-	-	-	8.659	2	<.025*
6	0.089	1	>.500	-	-	-	-	-	-
7	12.58	1	<.005*	-	-	-	-	-	-
8	1.177	1	>.100	0.074	1	>.500	6.773	1	<.010*
9	-	-	-	4.803	1	<.050*	0.635	1	>.100
10	0.039	1	>.500	0.057	1	>.500	0.004	1	>.900
11	1.150	1	>.100	-	-	-	10.29	1	<.005*
12	-	-	-	-	-	-	1.165	1	>.100

\* significantly different at .05 level

the genotypic frequencies in population 9 were significantly different from Hardy-Weinberg expectations (Table 5).

The PGM-2 locus had two of the twelve populations fixed for the "b" allele; all other populations had at least two alleles present (Table 4). Population 4 was unique in having all three of the PGM-2 alleles present. Chi square analysis indicated that the observed genotypic frequencies in populations 2, 5, 8 and 11 differed significantly from Hardy-Weinberg expectations (Table 5).

Data for the species (combination of all the populations) indicate that the fast (a) allele (Fig. 3) was at the lowest frequency at all three of the polymorphic loci (Table 6). However, there was considerable variation in the frequency of the "a" allele among populations. The other alleles also displayed variation in frequency (Table 6). Chi square contingency tests of both allelic and genotypic frequencies indicated that the observed frequencies deviated more than would be expected by chance alone (Table 7).

Estimates of genetic variation vary considerably among populations (Table 8). Direct count values of mean heterozygosity per individual (the mean number of loci that are heterozygous in the average individual) range from 1.12% in population 3 to 9.55% in population 10. Populations 1 and 12 indicate zero heterozygosity because no heterozygotes were detected even though both alleles were present in those populations. The chi square test for goodness of fit indicated no significant difference from Hardy-Weinberg expectations (Table 5). The difference seen between the calculated and observed heterozygosity was

Table 6. Allelic Frequencies at Polymorphic Loci.

Population	N	GOT-1		N	IDH-1		N	PGM-2		
		a	b		a	b		a	b	c
1	20	-	1.000	18	-	1.000	20	-	.150	.850
2	19	-	1.000	18	-	1.000	20	-	.400	.600
3	23	-	1.000	13	-	1.000	23	-	.870	.130
4	20	.275	.725	12	-	1.000	20	-	.775	.225
5	20	.425	.575	14	-	1.000	20	.300	.500	.200
6	20	.150	.850	13	-	1.000	20	-	1.000	-
7	20	.250	.750	10	-	1.000	20	-	1.000	-
8	19	.211	.789	18	.639	.361	19	.237	.763	-
9	19	-	1.000	20	.250	.750	20	.150	.850	-
10	20	.650	.350	20	.175	.825	20	-	.900	.100
11	20	.900	.100	19	-	1.000	20	-	.425	.575
12	20	1.000	-	20	-	1.000	19	-	.895	.105
Overall	240	.321	.679	195	.103	.897	241	.056	.712	.232

Table 7. Chi Square Contingency Test Results.

Genotypic Frequencies				Allelic Frequencies			
Locus	X <sup>2</sup>	df	Probability	Locus	X <sup>2</sup>	df	Probability
GOT-1	183.73	22	<.005	GOT-1	254.18	11	<.005
IDH-1	111.63	22	<.005	IDH-1	155.51	11	<.005
PGM-2	196.43	55	<.005	PGM-2	293.08	22	<.005

Table 8. Genetic Properties of the Populations Sampled.

Population	N	Polymorphic loci (%)	Mean heterozygosity per individual		Genetic diversity
			from frequencies (%)	direct count (%)	
1	20	11.1	2.83	0.00	.610
2	20	11.1	5.33	2.45	.971
3	23	11.1	2.52	1.12	.557
4	20	22.2	8.31	2.41	1.618
5	20	22.2	12.32	5.45	2.469
6	20	11.1	2.83	2.38	.610
7	20	11.1	4.17	1.30	.811
8	19	33.3	12.84	8.24	2.477
9	20	22.2	7.00	3.41	1.421
10	20	33.3	10.26	9.55	2.072
11	20	22.2	7.43	1.74	1.453
12	20	11.1	2.09	0.00	.485
Overall	242	33.3	8.81	3.13	2.455

probably the result of the heterozygote deficiency observed in nearly all populations (Tables 4 and 9). The mean proportion of loci polymorphic on a populational basis varied from 33.3% to 11.1%. The combined data result in a mean proportion of loci polymorphic in the species of 33.3% and a mean heterozygosity per individual of 3.13% (Table 8). Population genetic diversity was lowest in population 12 (.485) and highest in population 8 (2.477). Total genetic diversity in the species was 2.455.

Wright's  $F$  statistics were calculated as if the twelve sample localities were parts of a single large population. The results of these calculations are shown in Table 10. The  $\bar{F}_{is}$  values, which indicate the deviation from Hardy-Weinberg expectations, ranged in value from .7055 to .7401 over the three polymorphic loci. The mean  $\bar{F}_{is}$  value was .7340. The mean  $F_{it}$  correlation, which is the overall fixation index, is very high (.6835). The IDH-1  $F_{it}$  correlation appears significantly different from the GOT-1 and PGM-2  $F_{it}$  values (.4986 versus .7705 and .7813). The  $F_{st}$  values, which measure the differentiation of allelic frequencies, vary from .3496 at the PGM-2 locus to .5259 at the GOT-1 locus. The mean  $F_{st}$  value is .4241.  $F_{st}$  values derived from the  $F_{it}$  and  $\bar{F}_{is}$  values were significantly different from the  $F_{st}$  values calculated from the data. The  $F_i$  values for each population are shown in Table 10. The negative values (population 8 at the IDH-1 locus and population 10 at the PGM-2 locus) indicate an excess of heterozygotes. However, the chi square test for goodness of fit to Hardy-Weinberg

Table 9. Expected Frequency of Genotypes.\*

Population	N	GOT-1			N	IDH-1			N	PGM-2					
		aa	ab	bb		aa	ab	bb		aa	ab	bb	ac	bc	cc
1	20	-	-	20	18	-	-	18	20	-	-	0.4	-	5.2	14.4
2	19	-	-	19	18	-	-	18	20	-	-	3.1	-	9.8	7.1
3	23	-	-	23	13	-	-	13	23	-	-	17.3	-	5.3	0.4
4	20	1.4	8.2	10.4	12	-	-	12	20	-	-	11.9	-	7.2	0.9
5	20	3.5	10.0	6.5	14	-	-	14	20	1.7	6.2	4.8	2.5	4.1	0.7
6	20	0.4	5.2	14.4	13	-	-	13	20	-	-	20.0	-	-	-
7	20	1.1	7.7	11.2	10	-	-	10	20	-	-	20.0	-	-	-
8	19	0.8	6.5	11.7	18	7.2	8.5	2.3	19	1.0	7.1	10.9	-	-	-
9	19	-	-	19	20	1.2	7.7	11.1	20	0.4	5.2	14.4	-	-	-
10	20	8.3	9.3	2.4	20	0.6	5.9	13.5	20	-	-	16.1	-	3.7	0.2
11	20	16.1	3.7	0.2	19	-	-	19	20	-	-	3.5	-	10.0	6.5
12	20	20	-	-	20	-	-	20	19	-	-	15.1	-	3.7	0.2
Totals	240	51	51	138	195	9	22	164	241	3	19	137	3	49	30

\* frequencies calculated using Levene's (1949) formula for small samples.

Table 10. F Statistics for Ashmunella levettei.

	GOT-1	IDH-1	PGM-2	mean
All populations combined				
$\bar{F}_{is}$	.7401	.7565	.7055	.7340
$F_{it}$	.7705	.4986	.7813	.6835
$F_{st}^1$	.5259	.3967	.3496	.4241
$F_{st}^2$	.1170	-1.0591	.2574	-----
$F_i$ by population				
1	1.0000	1.0000	1.0000	1.0000
2	1.0000	1.0000	.5833	.8611
3	1.0000	1.0000	.6154	.8718
4	.6238	1.0000	.8588	.8275
5	.4885	1.0000	.6774	.7220
6	.2157	1.0000	1.0000	.7386
7	.7333	1.0000	1.0000	.9111
8	.6846	-.0838	.5669	.3892
9	1.0000	.4667	.6078	.6915
10	.1209	.1342	-.1111	.0408
11	1.0000	1.0000	.6940	.8980
12	1.0000	1.0000	1.0000	1.0000

1. corrected for sampling error

2. calculated from  $\bar{F}_{is}$  and  $F_{it}$

expectations, indicates no significant difference (Table 5). The  $F_i$  value of 1.000 indicates fixation of alleles.

The genetic similarity of the populations was calculated using Rogers' (1972) similarity index. The results of these calculations are shown in Table 11. The mean similarity of all populations (66 pairs) was a rather low .9003, with values ranging from .7968 to .9889. A value of 1.0000 indicates genetic identity.

A dendrogram, which indicates the genetic relationships of the twelve populations, was constructed (Fig. 4). Populations 3, 4, 5, 6, 7 and 9 form a cluster with a similarity of .9221. Populations 1 and 2 join this cluster at a similarity level of .9070. Populations 10, 11 and 12 form a cluster at a similarity level of .9184. The latter two clusters join at a similarity level of .8788, with population 8 joining this last large cluster at the .8669 level.

#### Morphological Results

Table 12 displays the data obtained from examination of shells from all twelve populations. The number of the population corresponds to its position in the Huachuca Mountains on a latitudinal basis, population 1 being the northern most population. The diameter of shell data indicated a size cline, with the largest specimens at the south end of the Huachucas (populations 10, 11, 12) and the smallest specimens at the north end of the range (populations 1, 2, 3). The height of shell data indicate the same cline. The data for number of whorls also show a cline, but it is the reverse of the previously noted clines. That is, those specimens with the lowest number of whorls are located at the

Table 11. Rogers' Similarity Estimates between Populations.  
 Results based upon allelic frequencies at nine loci.

Population	1	2	3	4	5	6	7	8	9	10	11	12
1	1.0000											
2	.9722	1.0000										
3	.9200	.9478	1.0000									
4	.9000	.9278	.9589	1.0000								
5	.8902	.9127	.9150	.9513	1.0000							
6	.8889	.9167	.9688	.9611	.9210	1.0000						
7	.8778	.9056	.9577	.9722	.9321	.9889	1.0000					
8	.8312	.8475	.8828	.8962	.8787	.8960	.8983	1.0000				
9	.8849	.9121	.9565	.9196	.8912	.9389	.9278	.9238	1.0000			
10	.8250	.8528	.9050	.9250	.9155	.9139	.9250	.8767	.9048	1.0000		
11	.8694	.8972	.8506	.8917	.9090	.8528	.8639	.7968	.8148	.9000	1.0000	
12	.8061	.8339	.8861	.9061	.8965	.8939	.9050	.8185	.8463	.9411	.9367	1.0000

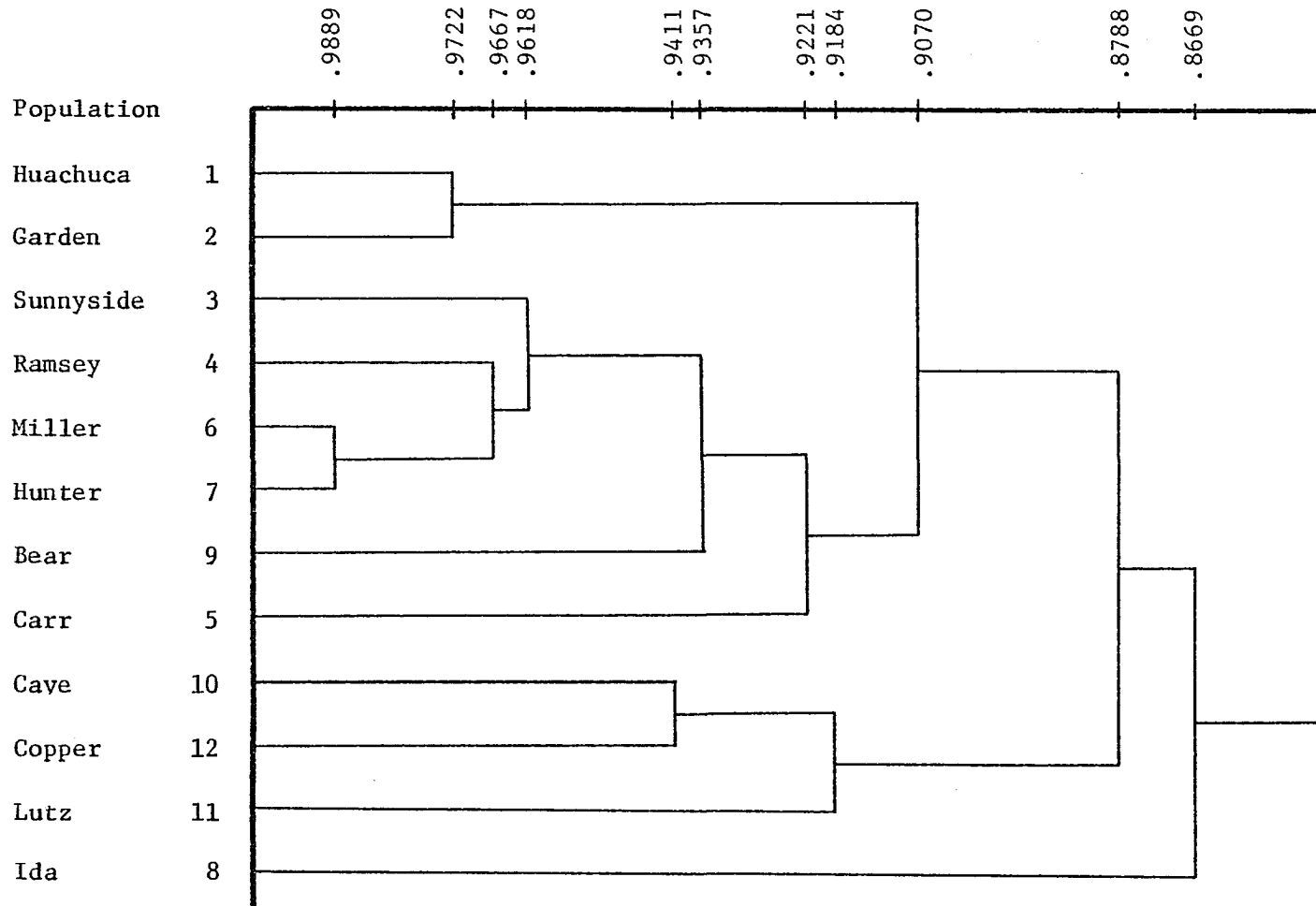


Figure 4. Dendrogram of Rogers' Similarity Estimates between Populations.

Genetic similarity on horizontal axis.

Table 12. Shell Measurements.

Mean, standard deviation and range.  
Measurements in mm.

Population	Diameter of shell	Height of shell	Number of whorls	Number of apertural teeth
1 (N=11)	13.31 (.489) 12.6 - 14.2	6.38 (.268) 5.8 - 6.6	6.57 (.197) 6 1/4 - 6 3/4	4.0 (---) ---
2 (N=9)	14.33 (.335) 13.8 - 14.8	6.70 (.292) 6.2 - 7.1	6.69 (.110) 6 1/2 - 6 3/4	4.0 (---) ---
3 (N=20)	12.38 (.443) 11.7 - 13.3	6.13 (.369) 5.5 - 6.9	6.29 (.203) 6 - 6 3/4	4.0 (---) ---
4 (N=26)	13.22 (.484) 12.1 - 14.0	6.16 (.297) 5.6 - 6.8	6.54 (.169) 6 1/4 - 6 3/4	4.0 (---) ---
5 (N=24)	14.05 (.568) 12.7 - 15.1	6.83 (.323) 5.8 - 7.5	6.40 (.207) 6 - 6 3/4	4.04 (.204)* 4 - 5
6 (N=22)	14.95 (.494) 13.8 - 16.2	7.52 (.489) 6.7 - 8.4	6.64 (.186) 6 1/4 - 7	4.0 (---) ---
7 (N=16)	15.11 (.532) 14.2 - 16.5	7.45 (.438) 6.9 - 8.1	6.53 (.256) 6 - 7	4.0 (---) ---
8 (N=20)	16.10 (.423) 15.3 - 17.1	7.83 (.451) 7.1 - 8.8	6.09 (.168) 6 - 6 1/2	3.4 (1.226) 0 - 4
9 (N=16)	16.08 (.607) 15.1 - 17.1	7.82 (.321) 7.1 - 8.3	6.05 (.136) 6 - 6 1/2	4.0 (---) ---
10 (N=21)	16.68 (.448) 16.1 - 17.6	8.02 (.483) 7.0 - 8.9	5.44 (.156) 5 1/4 - 5 3/4	0 ---
11 (N=21)	17.02 (.655) 15.5 - 18.1	7.98 (.570) 7.1 - 9.1	5.15 (.167) 5 - 5 1/2	0 ---
12 (N=25)	16.77 (.760) 15.1 - 18.2	7.93 (.340) 7.3 - 8.5	5.13 (.147) 5 - 5 1/2	0 ---

\* one of 24 had 5 teeth.

south end of the Huachucas, where as the specimens having the greatest number of whorls are found at the north end of the range. It should be noted that the populations can be placed into two groups, those with six or more whorls and those with less than six whorls. The number of "teeth" per shell was either four (populations 1 through 7, and 9) or zero (populations 10 through 12) except in population 8, in which the number of "teeth" varied from 0 to 4.

Analysis of variance tests (Table 13) indicate that (based on diameter of shell, height of shell and number of whorls) the samples examined (Table 12) were taken from different populations ( $P \ll .001$ ). Unfortunately, analysis of variance does not indicate which populations are significantly different from one another. The Student-Newman-Keuls test accomplishes this task (Fig. 5).

Visual comparisons of the internal anatomies of specimens from each population resulted in no discriminatory data. There was some variation in the absolute size of the various organs examined, however this variation was consistent with the variation in shell size. There were no qualitative or quantitative differences detected.

The habitat data are included in this section as a matter of convenience.

Based on the data in Table 14, it is apparent that Ashmunella levettei thrives best in northern exposures, high amounts of ground litter, high levels of shade, moist areas and is generally found under rocks. Correlations between genetic diversity and habitat parameters

Table 13. Results of Analysis of Variance Tests.

	F	df	Probability
Diameter of shell	74.14	8,96	< .001
Height of shell	36.30	8,96	< .001
Number of whorls	74.35	8,96	< .001

	Diameter of shell											
Population	<u>12</u>	<u>11</u>	<u>10</u>	<u>8</u>	<u>9</u>	<u>7</u>	<u>6</u>	<u>2</u>	<u>5</u>	<u>1</u>	<u>4</u>	<u>3</u>
	Height of shell											
Population	<u>10</u>	<u>11</u>	<u>12</u>	<u>9</u>	<u>8</u>	<u>7</u>	<u>6</u>	<u>5</u>	<u>2</u>	<u>1</u>	<u>4</u>	<u>3</u>
	Number of whorls											
Population	<u>12</u>	<u>11</u>	<u>10</u>	<u>9</u>	<u>8</u>	<u>3</u>	<u>5</u>	<u>4</u>	<u>7</u>	<u>6</u>	<u>1</u>	<u>2</u>

Figure 5. Results of Student-Newman-Keuls Tests.

Populations are arranged in order according to mean size, decreasing left to right. Those populations underlined by the same line are not significantly different (at the 5% level).

Table 14. Habitat Data.

Population	Elevation (in feet)	Exposure	Microhabitat	Ground <sup>1</sup> litter	Shade <sup>2</sup>	Moisture <sup>3</sup>	Area of collection <sup>4</sup>
1	6100	NNW	rocks	light	low	low	medium
2	6100	NNE	rocks	heavy	high	high	small
3	6200	flat-NE	logs-rocks	moderate	low	low	very large
4	7200	WNW	rocks	light	moderate	low	small
5	7025	NW-NE	rocks-logs	heavy	high	high	small
6	6050	NNW	rocks	heavy	high	high	very small
7	6200	NNE	rocks	light	high	moderate	small
8	7025	NW	rocks-logs	heavy	high	moderate	medium
9	6200	flat-NNW	logs-rocks	heavy	high	low	medium
10	6425	NNW	rocks-logs	heavy	high	moderate	medium
11	6300	NNE	rocks	heavy	high	low	very large
12	6050	WNW	rocks	heavy	high	high	very small

1. light = 25% covered; moderate = 50% covered; heavy = 75-100% covered.

2. low = 25% shaded; moderate = 50% shaded; high = 75-100% shaded.

3. low = water in winter only; moderate = water 1/2 of year; high = permanent water.

4. small = 30 X 50 feet; medium = 30 X 100 feet; large = 30 X 200 feet.

were all non-significant except for elevation. The correlation between elevation and genetic diversity was .8057.

The vegetation found at the collection localities is shown in Table 15. Examination of the data indicates that four species of trees (Silver Leaf Oak, Arizona Oak, Pinyon Pine and Apache Pine were found at 58.3% of the localities. Every locality had at least one of these four species of trees present. Douglas Fir was also present at 58.3% of the localities, primarily at those sites located at the highest altitudes. In all, 22 species of plants were identified at the twelve localities, with the number at a given locality varying from 3 to 9.

Table 15. Plant Species Present at the Collection Localities.

	Population	
1	++	+
2	++	+
3	++	+
4	++	+
5	+	+
6	+	+
7	+	+
8	++	+
9	++	+
10	++	+
11	++	+
12	+	+
	Silver Leaf Oak	
	Arizona Oak	
	Gambel Oak	
	Pinyon Pine	
	Apache Pine	
	Douglas Fir	
	Sycamore	
	Juniper	
	Madrone	
	Big Tooth Maple	
	<u>Rhus</u>	
	Willow	
	Box Alder	
	Arizona Walnut	
	Cottonwood	
	Mt. Mahogany	
	Silk Tassel	
	Agave	
	Beargrass	
	Horsetail	
	Barberry	
	Vine	
	Total species	

+ indicates presence

\* indicates major component

## CHAPTER 4

### DISCUSSION

Initial electrophoretic tests on Ashmunella levettei produced data that appeared comparable to data obtained by other investigators who have studied genic variation in land snails. Specifically, the observed percentage of loci polymorphic in A. levettei was 35.7 as compared to a mean of 43.7 for other species of land snails (Selander 1976). However, as the accumulated data were analyzed, they indicated what appeared to be a significant deficiency of heterozygotes (Tables 4 and 9). In a normally variable species, with allelic frequencies as determined for A. levettei, one would have expected more than 142 heterozygotes (Hardy-Weinberg expectations) rather than the 65 observed in this study. In order to examine more closely the observed genic variation and its possible causes, the following statistical analyses were conducted.

#### Genic Variation

Genotypic frequencies in each population and in the population as a whole were compared to Hardy-Weinberg expectations using the chi square goodness of fit test. This analysis tests the hypothesis that the observed genotypic frequencies are not significantly different from Hardy-Weinberg expectations.

Eight of twenty chi square tests for goodness of fit (Table 5) indicated a significant deviation from Hardy-Weinberg expectations in

individual populations. Chi square tests for goodness of fit, all populations combined (Table 16), indicated that the genotypic frequencies at GOT-1 and PGM-2 were significantly different from Hardy-Weinberg expectations. The genotypic frequencies at the IDH-1 locus were in agreement with expectations. Because a large proportion (> 40%) of the chi square tests indicated a significant deviation from expectations, it appears that evolutionary forces are operating with sufficient net effect to cause a significant deviation from Hardy-Weinberg expectations. It is important to remember that the deviations from expectations at the various loci were manifested as a large deficiency of heterozygotes. It should also be noted that in those populations that were polymorphic at the IDH-1 locus, in nearly all cases, the GOT-1 and PGM-2 loci were also in agreement with Hardy-Weinberg expectations.

Deviation from Hardy-Weinberg expectations can be caused by many factors, e.g., selection, mutation, gene flow and inbreeding. The magnitude of the observed deviations in Ashmunella levettei are so great that mutation is unlikely to be a significant factor. All of these populations are geographically isolated, hence gene flow is assumed to be zero. It seems reasonable to conclude that either selection or inbreeding (or both) is a factor contributing significantly to the observed heterozygote deficiency. The chi square test cannot indicate which factor is causing the deviation from expected genotypic frequencies; it can only indicate that some phenomenon is causing a deviation (Schaal 1975).

Table 16. Chi Square Values for Species Goodness of Fit.

Locus	$\chi^2$	df	Probability
GOT-1	18.728	1	<.005
IDH-1	1.232	1	>.35
PGM-2	58.670	2	<.005

For each locus, genotypic and allelic frequencies in each population were compared jointly with the frequencies in the species as a whole by a chi square contingency test. This analysis tests the hypothesis that the differences in frequencies among the twelve populations are no greater than among twelve random samples taken from the species as a whole.

The chi square contingency tests (Table 7) indicated that allelic and genotypic frequencies at all three polymorphic loci deviated significantly from those expected by chance alone. It appears that some evolutionary force or forces are causing the observed heterogeneity of frequencies. Workman and Niswander (1970) noted that when the chi square values vary considerably both among alleles at a locus and among loci, forces such as random genetic drift, errors in sampling, or selection may be the cause. Their chi square values varied from 0.58 to 120.41 (for genotypic frequencies) and 0.67 to 97.36 (for allelic frequencies). In both cases, the higher value is 150-200 times the lower value, suggesting that evolutionary forces are probably operating differently at each locus.

The variation among chi square contingency test values in this study is comparatively low (highs less than two times the lows), suggesting that whatever forces are operating to cause the observed heterogeneity of frequencies, they may be operating similarly at all loci.

#### F Statistics Analysis

The genetic structure of populations can be analysed by means of Wright's F statistics. This group of statistics provides a method

for partitioning an observed pattern of genetic variation. That is, the deviation from Hardy-Weinberg expectations ( $\bar{F}_{is}$ ), the degree of differentiation of allelic frequencies ( $F_{st}$ ), and the overall degree of allelic fixation ( $F_{it}$ ) can be quantified by describing the correlation between uniting gametes within groups, between groups and for all groups as a whole.

The mathematical theory for these statistical analyses is given by Wright (1943, 1951, 1965) and Nei (1965). In this discussion, only the essential formulae and concepts will be mentioned.

If the allelic proportions at a diallelic locus are represented by  $p$  and  $q$  ( $p + q = 1$ ) and one  $F$  statistic ( $F_i$ ), called a fixation index, the observed genotypic frequencies can be represented by

$$p^2 + pqF_i = AA$$

$$2pq(1 - F_i) = Aa$$

$$q^2 + pqF_i = aa$$

where  $F_i = 1 - H/2pq$  ( $H$  = the heterozygosity at the locus involved) (Workman and Niswander 1970). Here  $F_i$  indicates the deviation from Hardy-Weinberg expectations due to the joint effect of all factors acting on the pattern of genetic variation, including inbreeding, selection and mutation. Note that if  $F_i = 0$  the above equations reduce to the familiar Hardy-Weinberg equation  $p^2 + 2pq + q^2 = 1$ . The above equations provide a method of comparing different populations in terms of their deviation from a theoretical model, i.e., a population at Hardy-Weinberg expectations.

When several populations are considered together, the weighted mean of the  $F_i$  is indicated by  $\bar{F}_{is}$  so that

$$\bar{F}_{is} = \sum_{i=1}^r (N_i/N) F_i$$

where  $r$  is the number of populations,  $N_i$  is the number of individuals in the  $i$ th population, and  $N$  is the total number of individuals in all populations. This  $\bar{F}_{is}$  can be shown to be the average correlation between uniting gametes relative to the gametic arrays of their own subdivisions. That is,  $\bar{F}_{is}$  gives the average deviation of the population genotypic proportions from Hardy-Weinberg expectations (Workman and Niswander 1970).

The degree of differentiation among population allelic frequencies can be expressed by the  $F$  statistic  $F_{st}$  so that

$$F_{st} = \frac{\sum \sigma_{p(s)}^2}{\sum p_t(1-p_t)}$$

where  $\sigma_{p(s)}^2$  is the weighted variance of each allele at a locus, and  $p_t$  is the frequency of the allele (all populations combined).  $F_{st}$  gives the correlation between random gametes within populations relative to gametes of all populations combined (Levin 1977). That is,  $F_{st}$  gives the ratio of the actual variance in gene frequencies among populations ( $\sigma_p^2$ ) to its limiting value ( $p_t(1-p_t)$ ) which would result under complete fixation at the same allelic frequencies as in the combination of all populations (Workman and Niswander 1970). A more precise estimate of  $F_{st}$  can be obtained by correcting for sampling error.  $F_{st}$  corrected

for sampling error ( $F_{st}'$ ) is  $F_{st} - 1/2N_t$  (Workman and Niswander 1970), and in this study  $F_{st}' = F_{st} - .0021$ .

The third F statistic is  $F_{it}$ ,  $F_{it} = 1 - H_t / 2p_t(1 - p_t)$  where  $H_t$  is the heterozygosity for all populations combined, and  $p_t$  is the frequency of p for all populations combined.  $F_{it}$  is the correlation between uniting gametes relative to the gametic array of all populations combined (Levin 1977), that is, the total fixation index.

The three F statistics are not independent. If the differentiation among populations is random,  $F_{it} = \bar{F}_{is} + (1 - \bar{F}_{is})F_{st}$ . Estimating  $F_{st}$  from the other F statistics is referred to as the indirect method. Note that if all  $F_i$ 's are zero, and therefore  $\bar{F}_{is}$  is zero,  $F_{it} = F_{st} = \sigma_p^2 / \bar{p}\bar{q}$ , and thus there will be an observed deviation from Hardy-Weinberg expectations in the species as a whole (observed as a deficiency of heterozygotes). This was first observed by Wahland (1928) and is commonly known as the Wahland effect. The effect arises because samples having different allelic frequencies are combined to give an overall allelic frequency. The Wahland effect causes an increase in the observed  $\bar{F}_{is}$  value.

Among the populations studied, the mean  $F_i$  values varied from .3892 to 1.0000, indicating a high degree of fixation. The mean  $F_i$  over all populations and all loci is very high, .7466. A mean  $\bar{F}_{is}$  of .7340 was calculated for the species as a whole, indicating an average heterozygote deficiency of approximately 73%. The mean  $\bar{F}_{is}$  for Rumina decollata, a self-fertilizing species of land snail, was .7378 (calculated from data in Selander and Hudson 1976) (Table 17). High values

Table 17. F Statistics for Rumina decollata.

$F_{st}$  values from Selander and Hudson (1976),  
 $F_{is}$  and  $F_{it}$  values calculated from their  
 data by the author.

Locus	$\bar{F}_{is}$	$F_{st}$	$F_{st}^*$	$F_{it}$
LAP-1	.7466	.292	.3141	.8262
Est-6	.7289	.290	.3544	.8250
Est-10	.7341	.278	.3528	.8279
Est-11	.7353	.301	.3075	.8167
6-PGD	.7378	.306	.3674	.8380
mean	.7378	.2937	.3392	.8268

\* calculated from  $\bar{F}_{is}$  and  $F_{it}$  values

such as these can be caused by one or a combination of forces, e.g., Wahland effect, inbreeding or selection.

Schaal (1975) stated that it is unlikely that the contribution to  $\bar{F}_{is}$  by the Wahland effect would be larger than the value of  $F_{st}$ . In general, the  $F_i$  values and the  $\bar{F}_{is}$  values are so large that a Wahland effect of the magnitude of the observed  $F_{st}$  values would not totally explain the observed deficiency of heterozygotes in the populations of Ashmunella levettei. Levin (1977) has noted that if the primary contributions to  $\bar{F}_{is}$  were gene flow restriction and the spatial heterogeneity of gene frequencies within populations (which can cause a large Wahland effect), one would expect populations with high values of  $\bar{F}_{is}$  to have the most genetic diversity, since the most subdivided populations would best be able to store genetic variability. In this study, the correlation between  $\bar{F}_{is}$  for each population and their respective genetic diversities was  $-.6532$ . It appears that the Wahland effect does not contribute significantly to the observed deficiency of heterozygotes.

Inbreeding is a term applied when the individuals which mate together are more closely related to each other than are random members of an indefinitely large population (Allard, Jain and Workman 1968). Factors that can lead to inbreeding are isolation by distance, with the tendency of close neighbors to mate, and preferential mating between similar genotypes (Carson 1967). The former factor is largely a

function of mobility. Inbreeding can have a substantial effect upon  $\bar{F}_{is}$  values because it causes a progressive loss of heterozygosity, with the final allelic frequencies (fixation) equal to the original frequencies but with no heterozygotes (Allard et al. 1968).

Selection can have differing effects on the value of  $\bar{F}_{is}$  depending upon where the selective forces are acting. If natural selection favors homozygotes over heterozygotes, a heterozygote deficiency will result, increasing the  $\bar{F}_{is}$  value. However, over time loci would become fixed for one allele or another (Levin 1977). It appears that this type of selection is not operating in Ashmunella levettei since only 44.4% of the polymorphic loci are fixed. If selection is in favor of heterozygotes, the  $\bar{F}_{is}$  would be reduced below that dictated by the breeding structure, but there is no test to determine if this type of selection is taking place.

Wright's  $F_{st}$  measures the degree of differentiation of allelic frequencies among populations. The populations of Ashmunella levettei exhibit a very high degree of differentiation (mean .4241).  $F_{st}$  values for Rumina decollata average .294 (Selander and Hudson 1976), and for Helix aspersa (intercity) .162 (Selander and Kaufman 1975). Although the expectation of  $F_{st}$  is the same for all loci (Levin 1977), there is considerable variation among loci within A. levettei (Table 10). Possible causes of the variation among loci could be chance or selection. The high  $F_{st}$  values can have many causes, e.g., genetic drift, founder effect, selection or inbreeding.

The assumption of random (chance) variation can be evaluated by comparing estimates of  $F_{st}$  obtained from two different sources (Data and  $\bar{F}_{is}$  and  $F_{it}$ ) (Workman and Niswander 1970) (Table 10). If the estimates are the same, it is generally concluded that variation of  $F_{st}$  values among loci is random. If the two estimates are not in agreement, the conclusion is that the observed variation is not the result of a random process. Among the loci of Ashmunella levettei the two estimates of  $F_{st}$  do not agree, suggesting that the variation among  $F_{st}$  values is not the result of chance.

Workman and Niswander (1970) noted that the founder effect can cause genetic differentiation. That is, the ancestral populations, which gave rise to the present individuals, may have been genetically differentiated. It is doubtful that there was much initial differentiation among the ancestors of present day Ashmunella levettei populations, which are thought to have migrated to the Huachuca Mountains from Mexico or New Mexico (Pilsbry 1940). However, as the snails spread throughout the Huachucas, the founder effect could have been responsible for some of the differentiation observed in present day populations.

Genetic drift is another of the factors that can cause a heterogeneous distribution of allelic frequencies. In this case, the allelic frequencies would be expected to be randomly dispersed about the mean frequency for the entire species. Examination of Table 6 suggests that the frequencies are not randomly dispersed. Nei and Imaizumi (1966) found that if differentiation occurs at random by means of genetic drift, the allelic frequencies of close localities tend to be more alike than

those of localities far apart. The data in Table 6 are more difficult to assess in this regard, but it tends to support the possibility of random differentiation. It seems reasonable to conclude that genetic drift has probably contributed to the observed heterogeneity of allelic frequencies, but not to a significant degree.

Inbreeding affects the  $F_{st}$  values because it can cause the differentiation of a previously large randomly bred population into a series of subpopulations or inbred lines (Carson 1967). Differentiation of allelic frequencies among the subpopulations can cause an increase of the  $F_{st}$  values.

One additional and very important force that can affect the differentiation of allelic frequencies is selection. In a large population with random mating, allelic frequencies are not expected to differ significantly in different parts of the population (Schaal 1975). However, differential selection among different populations, which might reflect a heterogeneous environment, can cause heterogeneity of allelic frequencies.

$F_{it}$ , the total fixation index, was very high, indicating a strong tendency toward allelic fixation. The mean for all loci among all populations was .6835, as compared to a mean of .8268 for Rumina decollata a self-fertilizing species of land snail (calculated from data in Selander and Hudson 1976). The  $F_{it}$  for the Monarch Butterfly was .019 (Eanes and Koehn 1978) and for Bluegill populations .346 (Avisé and Felley 1979).  $F_{it}$  has elements of  $F_{st}$  and  $\bar{F}_{is}$  in it and thus those

evolutionary forces that affect these latter two  $F$  statistics also affect  $F_{it}$ .

Based upon field observations, Ashmunella levettei is a cross-fertilizing species of land snail. It is usually found in small isolated populations that form a patchy pattern over its range. Because of climatic and physical considerations, migration between populations is thought to be virtually impossible. Hence it is assumed that there is no gene flow between populations.

The vagility of Ashmunella levettei is low compared to some other species of land snail (e.g., Helix aspersa). It is unusual not to find several individuals under the same rock or log, suggesting a behavioral preference toward crowding. It appears that parents and offspring may remain in close proximity throughout their lives, which may be ten years or more (Walton 1963, 1970). Small isolated populations, low vagility, and the crowding phenomenon are all factors that contribute to the tendency toward inbreeding.

It was noted previously, that those populations (8, 9 and 10) that were polymorphic at the IDH-1 locus were probably in agreement with Hardy-Weinberg expectations at all loci (Table 5). Those populations that were monomorphic at the IDH-1 locus were all fixed for the same allele (populations 1 through 7, 11 and 12). Populations 8, 9 and 10 are relatively close to one another at the southern end of the Huachuca Mountains. Populations 8 and 10 are located in two branches of the same canyon, while population 9 is located on the opposite side of a ridge from population 8.

It seems very unlikely that nine populations would all become fixed for the same allele via the action of random genetic drift or the founder effect. It also appears very unlikely that selection would have caused the disappearance of the "a" allele in the nine populations surrounding populations 8, 9 and 10 without also affecting these latter populations.

A more likely possibility is that at some time in the past all populations were monomorphic at the IDH-1 locus. A new allele was probably created as the result of a mutation. If selection was in favor of the new allele, the frequency of that allele would have increased. Eventually, individuals carrying the new allele could have migrated to the other two populations that presently have it. As the climate changed and became dryer, migration to other populations was not possible, isolating the new allele in the three populations.

Bodmer and Parsons (1960) noted that in populations with random mating, selection must be in favor of the heterozygote in order for a new allele to increase in frequency. However, if there is inbreeding in the population heterozygote advantage is not necessary because the inbreeding would facilitate the establishment of the new allele.

#### Phylogeny and Taxonomy

Studies of the morphological variation of the shells from the populations sampled for this study have indicated a taxonomic problem among the subspecies levettei, bifurca and angigyra. These three subspecies are distinguished on the basis of size of shell, shape of the parietal tooth and the presence or absence of an accessory ridge

branching from the distal end of the parietal tooth. Based upon the type descriptions of these three subspecies (Pilsbry 1940), it is possible to segregate a mixture of specimens into groups that would represent each subspecies. However, each of the eight populations that might be considered one or the other of these subspecies has specimens that fit the description of each of these taxa. This study did not locate any populations that could be reliably identified as belonging to just one of these subspecies.

Because of the difficulty of correctly placing some of the populations into a currently recognized subspecies, new subspecific groups were formed. The criteria used to place a population into one of these new groups were (1) the number of apertural teeth and (2) the number of whorls in the shell. All other meristic characters were overlapping and formed north-south clines. The new subspecific groups are shown in Table 18. The subspecies heterodonta is unchanged in this new scheme, as is the subspecies varicifera. The subspecies levettei is composed of all remaining populations, the subspecies bifurca and angigyra are here synonymized with levettei.

Allelic frequency data from electrophoretic studies provide an excellent method to measure genetic similarity or distance among populations (see Avise 1975). There are a number of different methods available that summarize multiple locus allelic frequency data into a number that indicates the degree of similarity between a pair of populations. This study utilized Rogers' (1972) similarity measure because of its availability and widespread use in other studies. This

Table 18. Genetic Properties of the Subspecies of  
Ashmunella levettei.

Subspecies	<u>levettei</u> <sup>1</sup>	<u>varicifera</u> <sup>2</sup>	<u>heterodonta</u> <sup>3</sup>
N	161	60	19
Polymorphic loci (%)	33.3	33.3	33.3
Mean heterozygosity (%)	2.21	1.76	8.24
Genetic diversity	1.92	1.77	2.48
Mean genetic identity	.929	.926	1.000
$\bar{F}_{is}$	.8194	.6442	.3876
$F_{st}^4$	.2695	.1831	.0000
$F_{it}$	.6780	.4693	.3878

1. levettei is comprised of populations 1 - 7 and 9
2. varicifera is comprised of populations 10 - 12
3. heterodonta is population 8
4. corrected for sampling error

coefficient of genetic distance measures the average geometric distance between allelic frequency vectors on a scale from 0 to 1 (Allendorf 1975). Rogers' coefficient is defined as

$$D = \sum_{i=1}^r \left[ \frac{1}{2} \sum_{j=1}^{A_i} (P_{ijx} - P_{ijy})^2 \right]^{1/2}$$

where  $L$  is the number of loci,  $A_i$  is the number of alleles at the  $i$ th locus, and  $P_{ijx}$  and  $P_{ijy}$  are frequencies of the  $j$ th allele at the  $i$ th locus in populations  $x$  and  $y$  respectively. Genetic similarity is defined as  $1 - D$ .

Numerical taxonomic methods of cluster analysis were applied to the genetic similarity data obtained for the three subspecies to give a better visual presentation of genetic relationships. The unweighted average linkage method was used to construct the dendograms presented in this study. Sneath and Sokal (1973) have concluded that the average linkage methods (weighted or unweighted) provide the most accurate portrayal of data. The use of such a dendogram in making phylogenetic inferences relies upon the assumption that the rate of gene substitution per unit length of time is constant in all evolutionary branches.

A dendogram constructed for the new groups (Fig. 6) indicates that levettei and varicifera are more closely related to each other than heterodonta is to either one of them. The subspecies heterodonta is thought to be the result of crosses between toothed and untoothed individuals (Pilsbry 1940). The variable number of apertural teeth is morphological support for this hypothesis. Further support can be seen

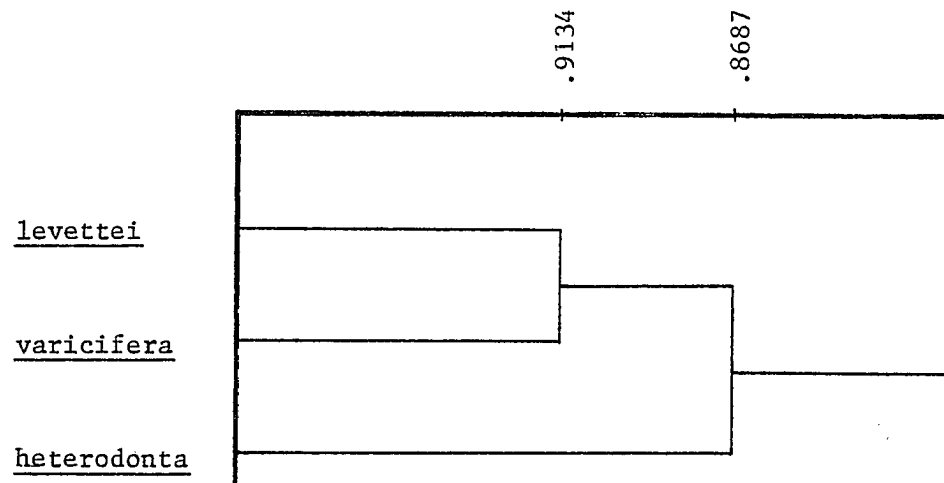


Figure 6. Dendrogram of Rogers' Similarity Estimates between Subspecies.

Genetic similarity on the horizontal axis.

in Tables 8 and 10. Population 8 (heterodonta) has the highest genetic diversity, and the mean heterozygosity per individual is second highest. In addition, the mean fixation index ( $\bar{F}_i$ ) is the second lowest. All of these observations are consistent with what might be expected from "hybrid vigor." The genetic similarity (I) values place heterodonta closer to levettei than to varicifera (.9007 versus .8367), which would be expected on the basis of shell morphology. The inconsistency between the morphological classification and the scheme indicated in the dendrogram (Fig. 5) is probably due, primarily, to the PGM-2 locus. The levettei and varicifera groups have the "c" allele at that locus, where as heterodonta does not.

The dendrogram of the individual populations (Fig. 4) indicates three groups. One group is a large loosely connected combination of eight populations (1 through 7 and 9) all of which are similar morphologically (average genetic identity .93). The second group is comprised of populations 10 through 12 (average genetic identity .93). The genetic identity values among these three populations varied less than among those populations comprising the first group. These three populations are also quite similar morphologically. The third "group" was population 8, which joins the other groups at the lowest level of similarity. The morphological distinction of population 8 is the variable number of apertural teeth. The mean genetic similarity among all populations was comparatively low, .9003. Mean genetic similarity for populations of Helix aspersa was approximately .97 (Selander and Kaufman 1975). Among populations of the bathyal gastropod Bathybembix bairdii

(Dall 1889) the mean genetic similarity was .99 (Siebenaller 1978), and among populations of Tridacna maxima Röding 1798, .97 (Campbell, Valentine and Ayala 1975). The low genetic similarity values (.8788 and .8669) at which the three groups of Ashmunella levettei populations are connected suggest that all of these populations may not belong to a single species.

The geographical distribution of these three groups is non-overlapping (Fig. 1), with the heterodonta population located between the varicifera and levettei groups. In addition, each of these groups has one or more distinguishing morphological characteristics. The varicifera group has no apertural teeth and less than six whorls in the shell. The levettei group has four apertural teeth and more than six whorls in the shell. The heterodonta population also has more than six whorls in the shell, but the number of apertural teeth varies from zero to four.

#### Summary and Conclusions

Based upon the observed allelic frequencies among the populations of Ashmunella levettei, it appears that there is a large heterozygote deficiency. In addition, it appears that the observed heterogeneity of allelic frequencies is significantly different from random expectations. The F statistics ( $\bar{F}_{is}$ ,  $F_{st}$ ,  $F_{it}$ ) partition the observed genetic variation so that the various evolutionary forces that affect genetic variation can be studied.

Studies of the  $\bar{F}_{is}$  values indicate that the observed heterozygote deficiency has several contributing factors, the Wahlund effect,

selection and inbreeding. The Wahlund effect does not appear to be the major contributor to the high  $\bar{F}_{is}$  values. The major contributor appears to be inbreeding, which causes an increase in the frequency of homozygotes at the expense of heterozygotes. However, if inbreeding is common one would expect the heterozygosity within these populations to be nearly nonexistent, with various alleles fixed in each population. This is not the case, hence it must be concluded that if inbreeding has been common in these populations some force must be maintaining the observed level of heterozygosity. The most likely force is selection in favor of the heterozygote.

Studies of the  $F_{st}$  values indicate that the observed degree of differentiation is probably caused by a combination of inbreeding, founder effects, drift and selection. The effects of drift and the founder principle are probably not of great significance. Inbreeding is thought to induce the differentiation of a large population into a series of subpopulations or inbred lines. This effect is followed by differentiation among subpopulations and reduction of genetic variability within each small subpopulation. Hence, inbreeding is probably the major cause of the observed genetic differentiation among the populations of Ashmunella levettei.

The  $F_{it}$  values are high, indicating a strong tendency toward allelic fixation. Again, inbreeding is probably the major cause.

Thus, at all three levels of population structure studied, it appears that the primary cause of the observed genetic variation is non-random mating, that is, inbreeding.

It was noted that a relatively recent mutation at the IDH-1 locus is isolated in only three populations. The isolation could be because of changing climatic conditions, which prevented further spread of the new allele.

There is an identification problem among some of the subspecies of Ashmunella levettei. In some populations, various specimens fit the descriptions of three different subspecies (levettei, bifurca and angigyra). Morphological studies of samples collected from eight populations have resulted in the conclusion that the subspecies bifurca and angigyra should be synonymized with the nominate subspecies A. 1. levettei. The genetic similarity data support the above conclusion.

Studies of the morphological and genetic similarity data have resulted in the conclusion that the subspecies A. 1. varicifera should be elevated to species rank, Ashmunella varicifera (Ancey 1901). The latter species is distinguished from A. levettei by having shells with no apertural teeth, and less than six whorls. In addition, the geographical ranges of these two species abut one another but no hybrids have been found (with the possible exception of the heterodonta populations which occur in only three known locations).

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